Formation of MTBE–DNA Adducts in Mice Measured with Accelerator Mass Spectrometry

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ABSTRACT: Methyl *tert*-butyl ether (MTBE) is a gasoline oxygenate and antiknock additive substituting for lead alkyls currently in use worldwide. Previous studies have shown that MTBE at very high doses induces tumors in rodents. The aim of the present study was to examine directly the binding ability of MTBE onto DNA, demonstrating its potential genotoxicity. MTBE–DNA adducts and their decay kinetics in mice have been measured by using doubly ^{14}C-labeled MTBE with an advanced, ultrasensitive technique: accelerator mass spectrometry (AMS). It was found that MTBE definitely formed adducts with DNA in mouse lung, liver, and kidney in a log/log linear dose–response relationship. The distribution sequence of DNA adducts in these tissues is: lung > liver > kidney. The level of MTBE–DNA adducts peaked at 12 h postadministration in the lung and peaked at 6 h postadministration in the liver. Then the adducts declined rapidly until 5 days postadministration and thereafter declined much more slowly. To our knowledge, this is the first report on DNA adduction with MTBE *in vivo*. The mechanism of the formation of MTBE–DNA adducts also is discussed. © 2005 Wiley Periodicals, Inc. Environ Toxicol 20: 397–401, 2005.

Keywords: methyl *tert*-butyl ether (MTBE); DNA adduct; accelerator mass spectroscopy (AMS); genotoxicity

INTRODUCTION

Methyl *tert*-butyl ether (MTBE) is a gasoline oxygenate and antiknock additive that substitutes for lead alkyls in worldwide use because it is cheaply produced and distributed, has a low boiling point and blending vapor pressure, and is easily blended without phase separation in gasoline (Suflita and Mormile, 1993; Mormile et al., 1994). It leads to increased oxygen content in gasoline, resulting in the reduction of carbon monoxide and hydrocarbon emissions from motor vehicles. In 2001 the total worldwide yearly production of MTBE from 173 plants was 22.5 millions tons (Zou, 2003), and minor growth was forecast for later years. As MTBE is a very water-miscible and volatile compound and its biodegradation in groundwater is slow (Squillace et al., 1997), the adverse effects of human exposure to MTBE have aroused considerable concern.

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Carcinogenicity tests have appeared to be positive for MTBE at high doses in a variety of species and target organs, inducing renal tumors and testicular tumors in male rats and liver tumors in female mice (Belpoggi et al., 1995; Bird et al., 1997). Therefore, it was classified as a possible human carcinogen by the U.S. Environmental Protection Agency (EPA) in 1997 (Caprino and Togna, 1999). But in 2002 the European Centre for Eco-toxicology and Toxicology of Chemicals (ECETOC) concluded, "The risk characterization for MTBE does not indicate concern for human health with regard to current occupations and consumer exposures" (ACFA and EFOA, 2002). MTBE also has been tested for mutagenicity both *in vivo* and *in vitro*, with negative results (Kado et al., 1998; Ahmed, 2001). Yet Lee et al. (1998) reported DNA strand cleavage in rat lymphocytes following exposure to MTBE. Zhou et al. (2000) reported that MTBE damaged the DNA of rat primary hepatocytes at a high concentration (1000 μg/mL); however, the damage was repairable. The doses used in these tests were all much higher than the actual dose of human exposure to MTBE.

When genotoxicity assays and carcinogenicity experiments of a chemical show an inconsistent combination of positive and negative results, direct investigation of DNA adduction is necessary or very helpful for assessing whether the test compound is a genotoxin (Phillips et al., 2000). So far, no published study has investigated MTBE bound with DNA *in vivo*, especially at a low dose.

Accelerator mass spectrometry (AMS), an ultrasensitive analytical method for DNA adduct measurement developed more than 10 years ago, has an extremely low detection limit: 1 adduct per 10^{12} nucleotides (Wang et al., 2001). The very high sensitivity of AMS make it possible to examine a variety of chemical xenobiotics at a low level of environmental exposure (Garner, 2000; Wang et al., 2001).

In the present study DNA adduction with MTBE in the lung, liver, and kidney of male mice was investigated by AMS. The decay kinetics of MTBE–DNA adducts following a single exposure also was examined in order to know how the DNA adducts were eliminated, mainly via DNA repair, versus time.

MATERIALS AND METHODS

Chemicals

Doubly ^{14}C-labeled MTBE [$(CH_3)_3{}^{14}CO{}^{14}CH_3$, purity $\geq$ 95%, specific activity 3.7×10^7 Bq/mmol] was purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of analytical grade or better.

Animal Treatment

The following animal experiments were conducted in accordance with national and institutional ethical guidelines for the protection of animal welfare. Male Kunming mice (about 25 g) were obtained from the Experimental Animal Center, Chinese Academy of Sciences (Beijing, China). Mice were randomized into 15 groups (8 mice per group) and housed in polycarbonate cages. Mice received food and tap water *ad libitum*.

^{14}C-MTBE dosing solutions were prepared by sequential dilution with saline (0.9% NaCl), and their concentration was determined using a liquid scintillation counter (Packard Tri-cab 2750 TR/LL, Packard Instrument Co., Meriden, CT, USA). In the dose–response study mice were administered doses of 0.95, 5.71, and 75.59 μg MTBE/kg body weight (bw) and 1.09 and 6.18 mg MTBE/kg bw by gavage (stomach intubation), using a dosing volume of 8 mL/kg bw. Mice were sacrificed at 6 h postadministration. The decay study in mice was performed following a single gavage exposure of 1.09 mg MTBE/kg bw. The mice were sacrificed after various intervals (2, 6, and 12 h; 1, 3, 5, 8, 11, 15, and 21 days) postadministration. A group of mice exposed to saline solution alone via gavage was used as the control (or background) group. The liver tissue samples of every two mice were merged into one sample; the lung tissue samples of every four mice were combined into one sample, as were the kidney tissue samples.

Extraction of DNA

DNA was extracted from these tissues and purified using a long protocol given by Gupta (1984). To guarantee the purity of DNA, only DNA with a 260/280 nm absorbance ratio of 1.82 + 0.05 was used. The specifically designed checking experiments indicated that this protocol, which was used to separate DNA, did exclude any contamination from exotic unbound ^{14}C (Wang et al., 2004).

AMS Sample Preparation and Measurement

To be measured by AMS, DNA samples must be converted to graphite, which was accomplished according to Vogel's protocol (1992). Conversion consisted of oxidation of DNA to CO_2 by CuO and further reduction of CO_2 to a graphite sample by Zn and TiH_2 in sealed tubes in a vacuum conversion system (courtesy of the Department of Earth System Science, University of California–Irvine, Irvine, CA, USA). Then the graphite samples were used as the ion sources for AMS measurement.

The samples were measured on the 2× 6 MV EN Tandem AMS facility at the Institute of Heavy Ion Physics, Peking University (Chen et al., 1990), and the results are given as the $^{14}C/^{12}C$ ratio with the unit of modern carbon (MC) (1 MC equals to 5.9×10^{10} ^{14}C/g of carbon, that is, 97.9×10^{-18} mol of ^{14}C/mg of carbon). This AMS system can measure the $^{14}C/^{12}C$ ratio with a sensitivity of 7×10^{-15} and an instrument precision of 1%–4%. Assuming

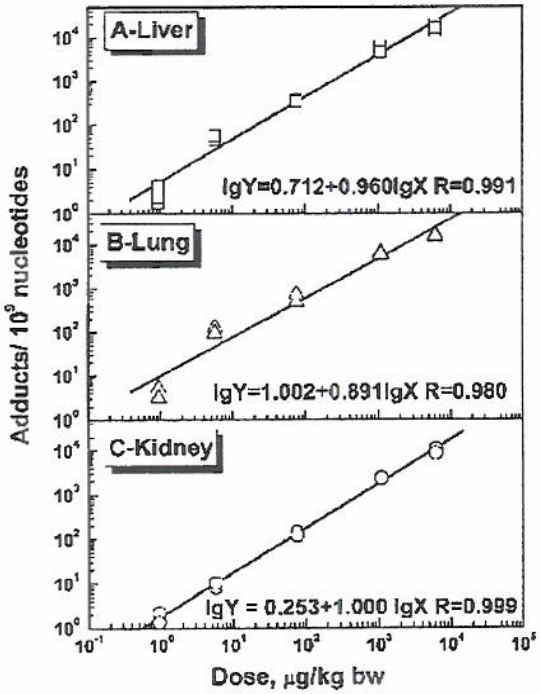

Fig. 1. Dose–response of ^{14}C-MTBE binding to DNA in the mouse liver, lung, and kidney at 6 h postadministration (2–3 parallel samples).

that the total ^{14}C contribution originates from the purified DNA itself, DNA adduct levels were then calculated using the $^{14}C/^{12}C$ ratios based on some fundamental constants [$T_{1/2}$ of ^{14}C – 5730 a, 1 MC – 5.9×10^{10} ^{14}C/g C, carbon content in DNA 30% (w/w), 1 μg of DNA – 3240 pmol of nucleotide]. Graphite standards prepared from Chinese glucose (Chinese standard, 1.362 MC) by the same sample preparation system were used as a monitor of any ^{14}C cross-contamination. The internal graphite standard oxalic acid-1 (No. SRM-4990, U.S. National Bureau of Standards) was used for normalizing the $^{14}C/^{12}C$ ratios in the AMS measurement.

RESULTS AND DISCUSSION

Figure 1 shows the dose response of MTBE–DNA adducts in the liver, lung, and kidney of male mice. All DNA adducts increased with an increasing dose in a log/log manner. Although we were unable to obtain structural information about the adduct, AMS measurement indicated that MTBE did bind with DNA in the mouse lung, liver, and kidney via its metabolites. The order of adduct concentrations in different tissues at any dose point was lung > liver > kidney, except after exposure to the highest MTBE dose of 6.18 mg/kg bw.

Generally, there are two possible routes for human exposure to MTBE: by inhalation (ACFA and EFOA, 2002) and via drinking water including ingestion, inhalation, and dermal absorption of contaminated water because of the easy contamination of groundwater and domestic water supplies (Davis and Powers, 2000). The estimated mean dose of the population via water is 1.4 μg MTBE/kg bw/day (Brown, 1997). Correspondingly, the lowest dose administered in this experiment was 0.95 μg MTBE/kg bw, leading to 2.76 + 1.27, 4.17 + 1.46, and 1.77 + 0.61 adducts per 10^9 nucleotides in the liver, lung, and kidney, respectively. The conclusion that MTBE can form adducts with DNA in mice after a single administration in a wide range of doses with a good linear correlation has not yet been reported in literature. In such a low dose of 0.95 μg MTBE/kg bw, the measured number of adducts is considered sufficiently high to possibly imply a high risk of carcinogenesis from MTBE.

Figure 2 shows that the level of MTBE–DNA adducts in the lung increased rapidly till 12 h after MTBE exposure. The adduct level reached the maximum at about 12 h postadministration. After that, the number of DNA adducts decreased rapidly. Then the curve declined with a much lower rate after a 5-day exposure.

Similarly, Figure 3 shows the decay curve (time course curve) of MTBE–DNA adducts in the liver, which peaked at 6 h postadministration. After that, the number of adducts decreased rapidly until 5 days, then declined slowly. The adduct level in the lung was larger than that in the liver. The number of adducts in the lung DNA was about 4.6-fold of that in the liver DNA at 504 h (21 days) postadministration. It is clear that MTBE–DNA adducts in both lung and liver can be detected over 21 days.

Previous pharmacokinetics studies have indicated that MTBE was rapidly absorbed by inhalation and ingestion in rats and mice and then eliminated via both exhalation and urine excretion (Dekant et al., 2001). The previous experiments using radiolabeled MTBE showed that excretion of

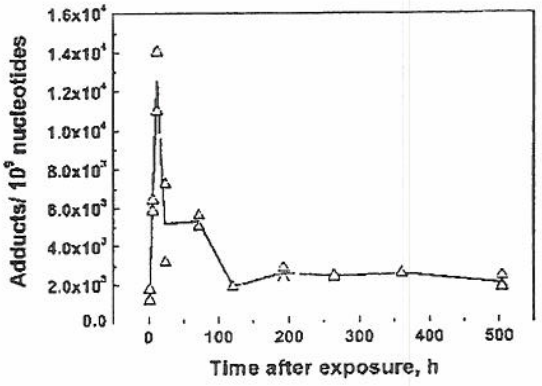

Fig. 2. Decay curve of MTBE–DNA adducts in the mouse lung. Mice were administrated 1.09 mg ^{14}C-MTBE/kg bw (2 parallel samples).

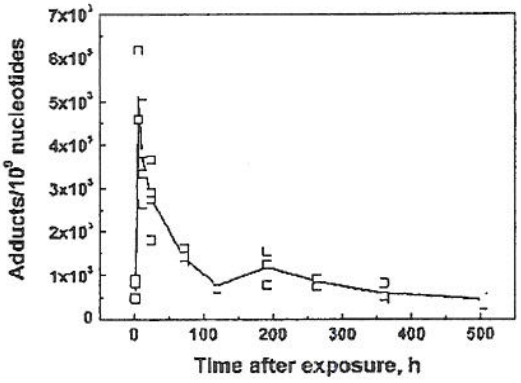

Fig. 3. Decay curve of MTBE–DNA adducts in the mouse liver. Mice were administrated 1.09 mg ^{14}C-MTBE/kg bw (2–4 parallel samples).

MTBE metabolites in mice via urine was essentially completed after 48 h. However, our study has shown that MTBE–DNA adducts persist even 21 days after a single dosing.

In rodents and humans, MTBE metabolized to *tert*-butyl alcohol (TBA) and formaldehyde via oxidative demethylation by the liver microsomes (Hong et al., 1997; Turini et al., 1998). Formaldehyde is most likely oxidized to formic acid in the liver. TBA is further oxidized to 2-methyl-1, 2-propanediol, and 2-hydroxyisobutyrate (Dekant, 2001). The doubly ^{14}C-labeled MTBE [$(CH_3)_3{}^{14}C—O—{}^{14}CH_3$] enabled us to detect all its metabolites that formed adducts with DNA. DNA adduction with a toxicant is an early and critical step in cancer initiation.

Generally, chemical assays do not always precisely predict the biological response, but an increase in adduct burden within a cell places the cell at greater risk of malignant mutation (Garner, 1998). In most cases DNA adduct level is positively correlated with the genotoxicity and hence the carcinogenicity of the chemicals (Otteneder and Lutz, 1999). Therefore, on the basis of our results, we suggest that MTBE is probably genotoxic.

Formic acid is a metabolic product of MTBE *in vivo*. Kryukov et al. (1997) preliminarily reported the formlyation of nucleosides via an esterification reaction between 5′-OH group of pentose and formic acid *in vitro*. Very recently, Chen et al. (2004) in our laboratory intensively repeated this experiment and confirmed the report of Kryukov et al. (1997). This indicates a possible pathway of DNA adduction with formic acid, that is, formic acid may covalently bind to the 5′-O position of pentose, thus resulting in cleavage of the DNA strand. Considering the similarity between MTBE–DNA adduct level and formic acid–DNA adduct level (Wang et al., 2004), it seems reasonable that the formation of the DNA strand at the 5′-O position of pentose by the metabolite formic acid is a potential pathway for the formation of MTBE–DNA adducts. Certainly, this proposition needs further experimental proof. However, one point worthy of attention is that the measured adducts might be partly contributed by the direct metabolic incorporation of formic acid via purines and pyrimidines into DNA (Boeniger, 1987). Very importantly, the nature of this incorporation pathway for formic acid makes MTBE nongenotoxic. In other words, MTBE would be less toxic to humans from a benign pathway.

To our knowledge, this is the first report on DNA adduction with MTBE *in vivo*. Using the ultrasensitive AMS technique and the radiotracer doubly ^{14}C-labeled MTBE, DNA adduction with the gasoline addictive MTBE in mice was studied. The results indicated that MTBE–DNA adducts definitely formed in the mouse lung, liver, and kidney, showing a good log/log linear dose–response profile. MTBE–DNA adduct level peaked within 12 h postadministration, then declined rapidly. Five days later the adduct level declined much more slowly.

The formation of the DNA strand at the 5′-O position of pentose by the metabolite formic acid is a potential pathway of the formation of MTBE–DNA adducts. The in-depth investigation of the mechanism by a combination of AMS and HPLC techniques is ongoing.

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